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AN OXALIC ACID PRODUCING PENICILLIUM.[1]

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Oxalic acid formation by fungi has received the attention of several investigators since de Bary[2] observed that the mycelium of *Sclerotinia sclerotiorum* became incrusted with an oxalate in its older stages. The most elaborate study of the problem was made by Wehmer.[3] His results were published in a series of twenty-three papers. Tabulated data showing oxalic acid production under various conditions were given for *Aspergillus niger* and *Penicillium glaucum*. No other molds were considered in detail.

Wehmer reviewed his own work and also that of other investigators up to 1906 in his chapter on the chemical action of Aspergillaceae contributed to Lafar's Handbuch der technischen Mykologie.[4] More recent literature contains several casual references to citric acid production in small quantities by various fungi but records no systematic study of the problem.

Wehmer states that the only acids produced by either *Aspergillus* or *Penicillium* in notable quantities are oxalic and citric. He concludes that investigators who have reported lactic, formic, and acetic acids did not work with pure cultures. Oxalic acid fermentation in its true sense, the production of free oxalic acid, according to Wehmer has been observed only in cultures of *Aspergillus niger*. *Aspergillus glaucus*, *Penicillium glaucum*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Rhizopus nigricans* produce oxalic acid only in traces and only as the media is maintained close to the neutral point.

Wehmer studied the various conditions which influence oxalic acid production. He found it was favored by low temperature, abundant carbohydrate food supply, the presence of an insoluble calcium salt as calcium phosphate or carbonate, and the presence of substances from which the mold can produce basic material such as organic nitrogenous compounds and salts of organic acids.

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[1] Published by permission of the Secretary of Agriculture.

[2] de Bary, A., *Bot. Ztg.*, 1886, xliv, 401.

[3] Wehmer, C., *ibid.*, 1891, xlix, 233.

[4] Lafar, F., Handb. d. technischen Mykologie, Jena, 2nd edition, 1905–07, iv, 242.

We have studied the oxalic acid production of twenty-three species of *Penicillium*. Many of these occur on the foodstuffs of man and of domestic animals under such conditions that oxalates might be produced. With the exception of the one species with which this paper is chiefly concerned it is doubtful if the quantity of oxalates formed would in any case be large enough to become a significant factor in food chemistry.

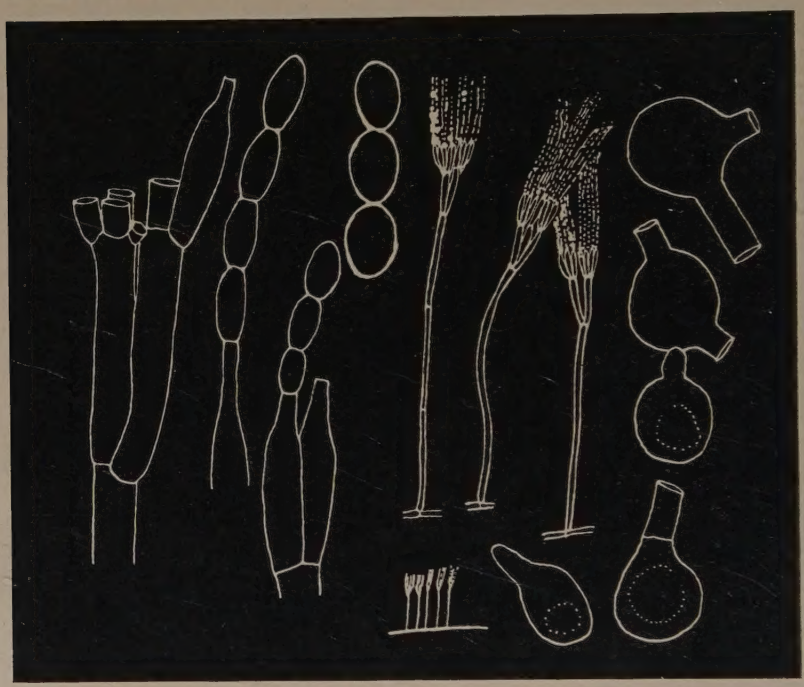

FIG. 1.

The three most important facts demonstrated are: (1) Oxalic acid is produced by many species of *Penicillium*. As a common product of their metabolism it may be likened to lactic acid among bacteria. (2) Oxalic acid is a transient and not an end-product of metabolism. (3) An undescribed *Penicillium* for which we propose the name *Penicillium oxalicum* should be added to the list of true oxalic acid ferments. A technical description of this organism with drawings is therefore given.

Description of Penicillium Oxalicum.

Colonies on Czapek's agar solution near ivy-green (Ridgeway XXXI) velvety, spreading widely with surface growth conidiophores only; reverse of colony pale yellow; agar uncolored or only slightly colored; conidiophores up to 200 μ long by 3.3 to 5.4 μ in diameter enlarged to 5 μ at apex; conidial fructification consisting of a single verticil of two to three branches 15 to 20 x 3.5 μ, appressed, bearing verticils of conidiiferous cells 10 to 14 x 2.5 to 3.5 μ in small parallel whorls which bear conidial chains in close columns which form visible masses of conidia easily broken free from the surface of the colony by tapping or jarring the glass; conidia at first cylindrical then elliptical from 2 x 3μ, 2 x 4 μ, 2 x 5 μ, up to 3.5 x 5 μ persistently elliptical, remaining in chains in alcoholic mounts swelling to 7 to 10μ in diameter and germinating by one or sometimes two tubes. No odor discernible.

Gelatin without sugar slowly liquefied more or less, with alkaline reaction. Produced rhombic crystals in prune gelatin. Grew richly at 37°C.

Shows a morphology of conidial apparatus resembling *P. italicum*, Wehmer, but differs widely from that species in appearance and habits. First found by T. P. Haslam in cultures from corn in Kansas. Later isolated from moldy corn and corn meal from many localities including Connecticut, Maryland, Virginia, and Illinois. It also occurs in soil cultures but evidently if it is a soil organism it is so well adapted to grow upon corn that it becomes a very common component of the flora of moldy corn and corn products.

Methods of Culture and Analysis.

The media employed were after the general formula of Czapek. The magnesium sulphate and potassium chloride were reduced because the magnesium tends to precipitate out the phosphate and the sulphate and chloride radicals add to the difficulties of separating the organic acids. Two media were used, one of which will be designated 3 per cent cane-sugar and the other 5 per cent cane-sugar. The inorganic salts used in each are shown in the following table.

	3 per cent cane-sugar.	5 per cent cane-sugar.
Sodium nitrate	1.0 gm. per 1000 cc.	3.0 gm. per 1000 cc.
Potassium nitrate	1.0 "	0.0 "
Potassium dihydrogen phosphate	0.5 "	1.0 "
Magnesium sulphate	0.01 "	0.25 "
Potassium chloride	0.01 "	0.25 "
Ferrous sulphate	0.01 "	0.01 "

Where the 3 per cent cane-sugar medium was used the mold was inoculated into 125 cc. of medium and 100 cc. of filtrate were analyzed. In all other cases the medium was filtered, the mycelium thoroughly washed with hot water, and the wash water added to the filtered medium. In no case did the mycelium after thorough washing yield a further quantity of oxalates even when digested with hydrochloric acid. The filtrate was then made alkaline with ammonia, heated almost to boiling, and a little magnesia mixture added. In this magnesia mixture chlorides were replaced by nitrates. This removes the excess of phosphates and a considerable quantity of pigment and other substances which would interfere with the purification of the oxalate. The oxalate was then precipitated as the calcium salt, dissolved in dilute hydrochloric acid, and reprecipitated. The twice precipitated oxalate was then dissolved in dilute sulphuric acid and estimated by titration with potassium permanganate. The purity of the calcium oxalate obtained by this procedure was established for nearly every mold studied by estimating the calcium oxide by direct ignition of the oxalate. When a calcium salt had been added to the medium the entire culture was thoroughly digested with warm hydrochloric acid before filtration and the removal of phosphates by magnesia mixture had to be omitted.

Examination of these data shows that *P. oxalicum*, culture number 103, under all conditions investigated far exceeded all others as a producer of oxalic acid. *P. italicum* which resembles *P. oxalicum* in morphology and also numbers 28 and 2467 which belong to the *Citromyces* group, produced very considerable amounts of oxalic acid when grown on media containing calcium carbonate. Wehmer[5] states that some of his *Citromyces* cultures formed oxalates as a secondary reaction on the citrate first formed, when cultivated in the presence of an excess of calcium carbonate.

Two cultures of *P. oxalicum* designated by numbers 103 and 3563.8 which had been derived from different sources were selected for further study. These were grown at 20°C. on 5 per cent cane-sugar media, and also on 10 and 20 per cent cane-sugar media in which sodium nitrate and potassium dihydrogen phosphate were proportionately increased. The other inorganic salts

[5] Wehmer, *Chem.-Ztg.*, 1909, xxxiii, 1281.

TABLE I.

Oxalic Acid Produced per 100 cc. of Medium, Expressed in $\frac{N}{10}$ cc.

Penicillium.*	Culture No.	3 per cent cane-sugar. 21 days at 25°.	3 per cent cane-sugar. 28 days at 25°.	5 per cent cane-sugar. 28 days at 25°.	5 per cent cane-sugar + $CaCO_3$. 28 days at 25°.	5 per cent cane-sugar. 14 days at 20°.	Per cent of CaO in calcium oxalate. Theory, 38.39.
Pinophilum	1	0.0	0.0	0.0	0.0	0.0	
Brevicaule	2	0.35	0.0	0.0	0.8	0.0	
Camemberti	5	11.6	21.7	31.0	52.1	22.0	38.42
Granulatum	9	11.7	15.1	31.6	32.3	21.4	38.41
Italicum	10	21.5	22.2	44.9	118.4	21.3	38.41
Luteum	11	17.5	12.5		15.6		38.32
Expansum	14	14.1	12.4	31.9	24.4	10.9	38.46
Citrinum	15	0.0	0.0	4.6	0.0	0.0	
Purpurogenum	17	0.0	0.0	0.0	0.0	0.0	
Roqueforti	18	21.0	26.2	30.4	48.8	11.6	38.51
Duclauxi	20	0.0	0.0	0.0	5.3	0.0	
Commune	23	14.6	19.0	32.9	15.5	16.8	
Chrysogenum	26	14.4	7.6				38.45
Stoloniferum	27	12.2	8.4	36.0	20.4	20.4	38.38
Divaricatum	34	0.0	0.0	0.0	0.0	0.0	
Spinulosum	45	0.0	0.0	0.0	0.0	0.0	
Notatum	102	0.0	0.0		38.1	6.2	
Oxalicum	103	54.1	53.0	35.2	288.0	86.6	38.39
Variabile	3551	0.0	0.0		19.2	10.2	
Not named	3553	11.2	13.4	14.1	38.7	25.4	38.27
Citromyces group	63	10.2	20.2			10.0	38.49
" "	2467	16.0	16.7	24.0	148.6	14.4	38.30
" "	28	17.4	14.6	13.6	122.0		38.29

* Designation of cultures is the same as that used by Thom, Cultural Studies of Species of Penicillium, *U. S. Dept. of Agriculture, Bureau of Animal Industry, Bull. 118,* 1910.

were as given for the 5 per cent cane-sugar media. At various intervals the acidity of these cultures was determined by titration with phenolphthalein as indicator and the oxalate radical estimated by precipitation as calcium oxalate and titration with potassium permanganate. For comparison *Aspergillus niger,* the oxalic acid ferment *par excellence* was included. Numbers 4, 5, and 6 were gently rotated daily to increase diffusion throughout

the media. It is difficult to accomplish this without submerging the mycelium, with resultant interference in continuous metabolism.

TABLE II.

Acidity per 100 cc. of Medium and Total Oxalate Radical, Expressed in $\frac{N}{10}$ cc.

	Per cent of cane-sugar.	5 days.		8 days.		12 days.		14 days.		21 days.		28 days.	
		Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.
1 *A.niger*	5	104.4	125.7	128.8	158.4	188.8	216.8	130.8	157.5	98.4	124.8	66.0	95.2
2 103.....	5	59.8	89.6	55.2	86.0	54.4	84.8	59.2	86.6	29.8	56.8	7.6	35.2
3 3563.8..	5	11.2	39.6	44.0	70.2	42.8	72.8	27.2	52.0	29.8	40.2	5.2	31.4
4 103.....	5	61.6	91.0	71.2	101.6	40.8	70.6	48.0	78.6				
5 103.....	10			67.5	117.1	97.5	151.4	79.0	132.8				
6 103....	20			72.0	119.0	106.5	179.3	124.0	195.8				

These results show that *P. oxalicum* though a less rapid producer of oxalic acid than *Aspergillus niger* is nevertheless a true oxalic acid ferment. It very quickly produces an acidity in media which under favorable conditions may reach 0.8 per cent free oxalic acid $(COOH.H_2O)_2$. The media from cultures of *P. oxalicum* are only slightly colored during the first eight to ten days and give a sharp end-point with phenolphthalein. Without the presence of an alkali carbonate to neutralize the acid formed, more than 1 per cent of oxalic acid accumulates as the free acid and as oxalates. Although the mold grows poorly and forms very few spores when grown upon media to which calcium carbonate is added, the yield of oxalic acid is greatly increased and may reach nearly 40 per cent of the weight of sugar employed. The results show clearly that oxalic acid is not an end-product but reaches a maximum point in eight to twelve days and then declines. If the media be agitated this maximum point is attained more quickly.

Penicillium oxalicum is very omniverous in habit. Its ability to produce oxalic acid from various sources of carbon was tested by growing it upon media made up exactly as the 5 per cent cane-sugar media except that the sugar was replaced by 5 per cent of the organic substance to be tested.

TABLE III.

Oxalic Acid Produced per 100 cc. on 5 per cent Medium, Expressed in $\frac{N}{10}$ cc.

Culture.	10 days at 20°.		15 days at 20°.	
	103.	3563.8.	103.	3563.8.
Glucose	105.6	78.0	87.8	41.6
Lactose	95.6	34.4	114.8	93.8
Potato starch	106.8	66.8	103.0	66.6
Glycerin	5.3	10.3	19.4	56.6
Peptone	89.3	58.8	117.6	80.4
Calcium lactate		•	15.2	8.6

Both cultures grew luxuriantly on all these media except glycerin and calcium lactate. The most striking cultural difference observed between these two cultures was their deportment on glycerin media. In all other cases 103 had showed itself more active than 3563.8, but on glycerin this was reversed. This was shown very plainly in the abundance of mycelium as well as in the actual production of oxalic acid. The ability of this organism to hydrolyze gelatinized starch is shown from the fact that it produced as much oxalic acid in ten days from starch as from a soluble sugar.

It has not been the purpose of the authors to indulge in any speculations concerning the mechanism of oxalic acid formation or its biological significance with regard to the metabolism of fungi. The chief interest of this paper lies in the fact that we have observed a *Penicillium* which forms oxalic acid as the chief product of its metabolism. This is an observation hitherto unrecorded in the literature of fungi.

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